UNIVERSITY OF PENNSYLVANIA

---

# CYTOCHROME C IN REGENERATING RAT LIVER AND ITS RELATION TO OTHER PIGMENTS

A DISSERTATION

IN PHYSIOLOGICAL CHEMISTRY

TO BE PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY TO
BE CONFERRED WHEN ALL OTHER RE-
QUIREMENTS HAVE BEEN FULFILLED

MARYLIZABETH WELLINGTON CRANDALL

IN COLLABORATION WITH DAVID L. DRABKIN

PHILADELPHIA
1946

UNIVERSITY OF PENNSYLVANIA

---

# CYTOCHROME C IN REGENERATING RAT LIVER AND ITS RELATION TO OTHER PIGMENTS

A DISSERTATION

IN PHYSIOLOGICAL CHEMISTRY

TO BE PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY TO
BE CONFERRED WHEN ALL OTHER RE-
QUIREMENTS HAVE BEEN FULFILLED

MARYLIZABETH WELLINGTON CRANDALL

IN COLLABORATION WITH DAVID L. DRABKIN

Reprint from

*The Journal of Biological Chemistry, Vol. 166, No. 2*

PHILADELPHIA

1946

R. UNIV.
BIBLIOTHEEK
LEIDEN

# CYTOCHROME *c* IN REGENERATING RAT LIVER AND ITS RELATION TO OTHER PIGMENTS

The development of satisfactory methods for the determination of cytochrome *c* in small amounts of tissue (1–3) has led to the accumulation of data upon the distribution and concentration of this important biocatalyst in various tissues of different species (1–7). Except for such information, nothing is known concerning the metabolism of cytochrome *c*. We regarded it desirable first to gain insight into the rate of production of this pigment. A quantitative "depletion technique" of the type used so successfully in Whipple's laboratory (8) for the study of hemoglobin production is not available for a constituent of practically all aerobic cells. As an approach in this direction we have applied the technique of partial hepatectomy and have secured data permitting the calculation of the rate of appearance of new cytochrome *c* in the regenerating liver.

The early literature upon the regeneration (restoration) of the liver has been reviewed by Fishback (9). Ponfick (10) appears to be the first to have studied liver restoration after partial hepatectomy. Interest in this experimental technique has been revived by the more modern, careful studies of Higgins (11), Brues (12, 13), and Marshak (14) and their collaborators. These investigators were concerned largely with the cytological features of the restoration process, which has been established (12, 13) as true hyperplasia (production of new tissue) in contrast to hypertrophy (enlargement of existing tissue).

Biochemical investigations of gaseous exchange as a measure of rate of glycolysis (15), riboflavin content (16), and content of enzymes such as catalase, arginase (17), and cytochrome oxidase (18) have resulted in the conclusion that regenerating or restored liver, following partial hepatectomy, and adult normal liver appear to be chemically indistinguishable. In contrast, embryonic and neoplastic livers have a high anaerobic glycolysis (15) and are low in certain essential biocatalysts (16–18). However, in none of this chemical work has attention been directed towards the quan-

titative aspects of the restoration process. The analytical data cannot be used to evaluate the rate of production, or other metabolic features, of the components measured. Approaches to the use of partial hepatectomy as a metabolic technique have been made in studies of vitamin A (19) and plasma proteins (20). The work on vitamin A, a very readily mobilizable component of liver (19), has been limited to analyses in a very short post-operative period (3 to 4 hours), and therefore cannot be related unequivocally to the restoration process. The investigation of Chanutin and colleagues (20) was confined to the effect of partial liver excision upon the plasma proteins, presumably with no simultaneous study of changes in the proteins or other constituents of the liver during restoration.

In applying the partial hepatectomy technique to the study of the metabolism of cellular components in the intact animal, it is essential that the experimental procedure be shown to be quantitatively reproducible. Data will be presented from albino rats of our colony fully confirming earlier demonstrations (11–13, 15) of the relative constancy of relationship in this species between liver mass and body mass, and particularly between excised lobes, remaining tissue, and total liver. This constancy of anatomical relationships in liver, which now appears established in the rat, supplies the necessary base-line for quantitative determinations. The surgical excision of rat liver tissue is simple and rapid. Owing to the distinctive multilobed character of the liver in this species (Fig. 1), the operative procedure may be aptly designated as *liver lobectomy*. The whole metabolic procedure is economical of time, since total regeneration (restoration) after removal of two-thirds of the liver occurs within 10 to 14 days in adult rats. Thus, the measurement of the restoration of tissue components in the regenerating liver of the rat as a metabolic technique has the desirable attributes of reproducibility, ease of application, and economy of time.

During the course of this work sufficient additional data upon the cytochrome *c*, myoglobin, and hemoglobin concentrations have been gathered to permit a reliable estimate and comparison of the total content in the body of the rat of these three main hemin derivatives. Such data are of interest from the standpoint of the respective metabolic rôles of the substances under consideration.

## *Experimental Methods*

*Analytical Procedures*—The concentration of cytochrome *c* in individual rat organs was determined by the direct micro spectrophotometric method of Rosenthal and Drabkin (3).[1] The total cytochrome *c* of the body (Table

[1] We again use the molecular weight of 13,000, corresponding to Theorell's value of 0.43 per cent for the iron content of pure cytochrome *c* (21). From their recently published data we conclude that Keilin and Hartree (22) have demonstrated that the biocatalytic activities of cytochrome *c* preparations with 0.34 and 0.43 per cent iron are

II) was obtained by a summation of values calculated from the analytical data for the individual organs of a representative 250 gm. male rat. The organ weights are approximations based upon three rats of this size from our colony.

The myoglobin was extracted by a quantitative volumetric application (with appropriate use of dilution factors) of Morgan's modification of Theorell's method (23). The isolation was carried to the stage of separation of hemoglobin and solution of the muscle pigment in 3 M phosphate buffer (Morgan (23)). The quantitative determination of the myoglobin in this solution was by either of two procedures. (1) The pigment was totally converted to cyanmetmyoglobin[2] by the addition of ferricyanide and cyanide, and the concentration determined spectrophotometrically, with the constant $\epsilon$ ($c$ = 1 mM per liter, referred to a molecular weight of 16,450, based on 0.34 per cent of iron, $d$ = 1 cm.) = 11.3 at wave-length 540 m$\mu$.[3] This procedure was the basis for our derivation of the total myoglobin content of the rat organism (Table III). (2) An alternative microprocedure, applicable to as little as 1 gm. of muscle relatively rich in myoglobin, or to 3 to 8 gm. of myoglobin-poor muscle (as in the present work on the rat), has also been used (Table III). In this method the myoglobin in the 3 M phosphate buffer (extracted as in Procedure 1) is transferred into aqueous, strongly alkaline pyridine. The pyridine layer separates by stratification, and the myoglobin is determined spectrophotometrically after the addition of solid dithionite ($Na_2S_2O_4$) as pyridine ferroprotoporphyrin (reduced pyridine hemochromogen), with $\epsilon$ ($c$ = 1 mM per liter, $d$ = 1 cm.) = 29.1 at wave-length 557 m$\mu$ (25) as a provisional constant. The analysis is carried out with our 3.4 cm. capillary cuvette-diaphragm technique (3). A more detailed account of these methods for myoglobin, developed by one of us (D. L. D.), will be presented in a later communication.

The hemoglobin concentration was determined spectrophotometrically as cyanmethemoglobin, with the constant $\epsilon$ ($c$ = 1 mM per liter, $d$ = 1 cm.)

---

proportional to their iron content (equivalent per atom of iron). We have difficulty in accepting the argument of the English investigators that their data suggest that cytochrome *c* with 0.43 per cent of iron (mol. wt. 13,000) is a degradation product (rather than a purification) of the "natural" cytochrome *c* with 0.34 per cent of iron (mol. wt. 16,450). Keilin and Hartree (22) appear rather to have established the high biological activity of the lower molecular weight cytochrome *c*.

[2] An analogue of cyanmethemoglobin, with practically identical spectroscopic properties (24).

[3] In the notation $\epsilon$ ($c$ = 1 mM per liter, $d$ = 1 cm.), $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration, $c$, is expressed in mM per liter, the depth, $d$, in cm., the intensity of incident radiation, $I_0$, is 1.0, and the intensity of transmitted radiation, $I$, is expressed as a fraction of unity. The value of the constant for the maximum of cyanmetmyoglobin has been established upon an iron basis by one of us in other work (24), details of which will be reported separately.

= 11.5 at wave-length 540 mμ (26). The total hemoglobin of the animal (Table III) was calculated from (gm. of hemoglobin per 100 ml./100) × (plasma volume in ml.)/(1 − hematocrit, written as a fraction of unity). The values for the concentration of hemoglobin and hematocrit were in good agreement with earlier extensive determinations of these quantities in the rat in our laboratory (27). The value of plasma volume is based upon a single determination with the dye T1824 (28), and should be regarded as an approximation sufficient for our present purpose.

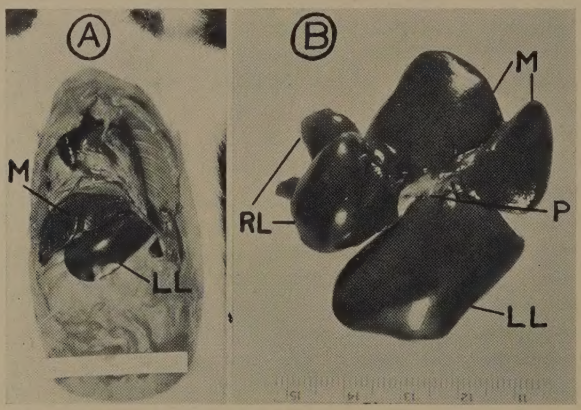


FIG. 1. Structural relationships of rat liver. *A*, liver *in situ*. *B*, whole liver excised, with notched median lobe turned back. *LL*, left lateral lobe, which presents first at the partial hepatectomy operation; *M*, median lobe; *RL*, right lateral and caudal lobes; *P*, pedicles of the left lateral and median lobes, to which ligatures are applied for excision of either the left lateral lobe alone or the left lateral plus the median lobe. The linear dimensions are indicated by the cm. scales.

*Partial Hepatectomy* (*Liver Lobectomy*)—Adult albino rats of 184 to 332 gm. in weight (averaging 150 to 200 days old) were employed. The animals of our laboratory colony are originally of Wistar Institute stock, inbred for many generations (22 years) under the supervision of Dr. J. H. Jones. The rats were fasted preoperatively for 18 to 24 hours. The surgical technique was simple, but complete asepsis was observed. After moderate ether anesthesia, enough to produce complete relaxation, the abdomen from the sternal region down was shaved and sterilized. The abdomen was then opened by means of a longitudinal, mid-line incision from over the xiphoid process caudally for about 3 to 4 cm. The desired left lateral or left lateral and median lobes were readily delivered. These lobes are on separate pedicles (*P*, Fig. 1) to which a hemostat was applied. A silk ligature was firmly tied around one or both pedicles beyond the hemostat, depending on whether the left lateral lobe alone (in most of our ex-

periments) or the left lateral and median lobes were to be removed. By division of the pedicle, the lobe or lobes were then excised and transferred to a small air-tight vessel (such as a weighing bottle), previously provided on its bottom with a piece of filter paper dampened with 0.9 per cent NaCl. (This provision is to prevent dehydration by evaporation before weighing the excised tissue.) The anesthesia was now usually stopped, and the abdomen closed by silk sutures, the peritoneum and muscle layer with a baseball stitch, and the skin layer separately with interrupted sutures about ¼ inch apart. The incision area was coated lightly with Whitehead's varnish. For the 1st postoperative day the animals were kept in a warm room (temperature about 27°). The whole surgical procedure took no more than 10 minutes and was free from mortality. However, some caution is advisable. Rat liver is rather friable, and must be handled gently. Also, care must be taken to avoid including a portion of the remaining right lateral lobe in the ligation. Bandages or adhesive tape should not be used during wound healing.

Since the terminology for the lobes of rat liver is not consistent, we follow that used by Higgins and Anderson (11). Fig. 1, *A* and *B*, is given for orientation.

As quickly as possible after the operation the excised liver lobe or lobes were rinsed with water, adhering fluid was blotted off with filter paper, and the tissue accurately weighed. The determinations of cytochrome *c*, dry weight, and protein-bound phosphorus were then carried out on accurately weighed aliquots. Dry weight was obtained by heating 50 to 60 mg. of minced tissue in micro crucibles for 2 hours at 100°, followed by cooling in a desiccator over $CaCl_2$. Protein-bound phosphorus as an index of cellularity was determined as described by Rosenthal and Drabkin (6).

After a period of 14 days, the interval of time which has been found (11, 12) adequate for complete restoration of the liver, the rats were sacrificed by stunning, followed immediately by decapitation. This procedure afforded moderately good exsanguination. The entire restored liver was removed, treated as above, and accurately weighed. Analytical data were secured not only on the regenerated liver, but also on the heart, kidney cortex, and skeletal muscle of each animal. During 13 days of the restoration process the rats were maintained either on a modified Steenbock stock diet (29),[4] adequate in protein, or on an enriched protein diet, the stock diet bolstered with casein.[4] The animals were then fasted for 1 day before the termination of the experiment. This was deemed necessary to assure com-

[4] Stock diet, ground yellow corn 74, linseed oil meal 16, crude casein 5, alfalfa 2, NaCl 0.5, $CaCO_3$ 0.5, and yeast 2 parts per hundred. This diet affords approximately 20 per cent of protein (personal communication from Dr. J. H. Jones). The enriched protein diet was the stock diet 80 parts plus casein (80 per cent protein) 20 parts. This mixture afforded approximately 32 per cent of protein.

TABLE I

*Concentration (C) of Cytochrome c in Micrograms per Gm. of Wet Weight of Tissue in Control Rats, Fasted 18 to 24 Hours*

| Rat No. | Sex | Body weight | Liver | | Heart | | Kidney cortex | | Skeletal muscle | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *C* | *W : D** | *C* | *W : D** | *C* | *W : D** | *C* | *W : D** |
| | | *gm.* | | | | | | | | |
| 1 | M. | 223 | | | | | 102 | | 61 | 3.79 |
| 2 | F. | 195 | | | 399 | 3.95 | 342 | 4.10 | 55 | 3.98 |
| 3 | M. | 179 | | | 528 | 4.45 | 413 | 3.56 | 93 | 3.90 |
| 4 | " | 272 | | | 600 | 4.30 | 337 | 3.67 | 86 | 3.78 |
| 5 | " | 437 | | | 414 | 4.68 | 106 | 4.39 | 97 | 3.84 |
| 6 | " | 362 | | | 443 | 4.55 | 349 | 4.10 | 93 | 4.15 |
| 7 | " | 361 | | | 206 | 4.24 | 181 | 3.82 | 64 | 3.89 |
| 8 | " | 384 | | | 508 | 4.05 | 390 | 3.95 | 60 | 4.01 |
| 9 | " | 415 | | | 377 | 4.18 | 460 | 3.82 | 128 | 3.78 |
| 10 | " | 283 | | | 434 | 4.45 | 408 | 3.97 | 113 | 3.76 |
| 11 | " | 334 | | | 455 | 4.36 | 385 | 4.23 | 83 | 3.81 |
| 12 | " | 227 | | | 494 | 4.52 | 309 | 3.97 | 131 | 3.94 |
| 13 | " | 291 | | | 495 | | 430 | | 137 | 3.76 |
| 14 | " | 231 | | | 490 | 4.19 | 533 | 4.02 | | 3.78 |
| 15 | | | | | 574 | 4.12 | 259 | 4.16 | 134 | |
| 16 | M. | 328 | | | 478 | 4.24 | | 3.76 | 125 | 3.90 |
| 17 | " | 232 | 296 | 3.03 | 429 | 4.08 | 319 | 4.17 | 135 | 3.82 |
| 18 | " | 300 | | | 397 | 4.27 | 490 | 4.17 | 147 | 4.05 |
| 19 | F. | 192 | 164 | 4.40 | 425 | 4.76 | 431 | | 114 | 4.38 |
| 20 | M. | 314 | 193 | 3.15 | 498 | 4.01 | 342 | 4.13 | 56 | |
| 21 | " | 306 | 273 | 3.36 | 478 | | 400 | 4.55 | 48 | 3.44 |
| 22 | " | 278 | 285 | 3.28 | 348 | 4.20 | 369 | 4.27 | 106 | 3.87 |
| 23 | " | 306 | 257 | 3.18 | 312 | 4.82 | | 3.98 | 98 | 3.89 |
| 24 | " | 292 | 329 | 3.33 | 474 | 4.66 | 347 | 4.42 | 114 | 4.04 |
| 25 | " | 297 | 260 | 3.07 | 467 | 4.26 | 358 | 4.00 | | 3.85 |
| 26 | " | 357 | 334 | 3.04 | 440 | 4.45 | 407 | 4.29 | 84 | 3.89 |
| H1† | F. | 184 | 96 | 3.77 | | | | | | |
| H2† | " | 190 | 159 | 3.46 | | | | | | |
| H3† | " | 198 | 131 | 3.38 | | | | | | |
| H4† | " | 201 | 105 | 3.34 | | | | | | |
| H5† | M. | 267 | 218 | 3.68 | | | | | | |
| H6† | " | 268 | 180 | | | | | | | |
| H7† | " | 221 | 181 | 4.01 | | | | | | |
| H8† | " | 288 | 268 | 3.43 | | | | | | |
| H9† | " | 332 | 290 | 3.37 | | | | | | |
| Mean ± s.e.‡..... | | | 223 ±18 | 3.43 ±0.09 | 447 ±16 | 4.34 ±0.05 | 352 ±21 | 4.07 ±0.05 | 98 ±6 | 3.89 ±0.04 |

* *W : D* = wet weight to dry weight ratio of tissue.

† Rats H1 to H9 are the partially hepatectomized series. These analyses are on the liver tissue excised at operation.

‡ Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

parable conditions of alimentation (which affects the composition of "storage" tissues like liver (30)) in the pre- and postregeneration periods.

*Results*

Our data upon the cytochrome *c* concentration of liver, heart, kidney cortex, and skeletal muscle of thirty-five "control" fasted rats are collected in Table I. The values for cytochrome *c* in liver lobes excised ("internal controls") in the partially hepatectomized series (Rats H1 to H9) are included. No difference in the cytochrome *c* content is apparent from the values obtained in liver from these animals and from non-operated, normal fasted rats (compare the analyses of Rats H1 to H9 with Rats 17 to 26). Similar wet weight to dry weight ratios, $W:D$, are also apparent in these two groups of animals. While it is convenient in the present work to express the cytochrome *c* concentrations on a wet weight basis, the $W:D$ ratios are supplied as an index of the water content of tissues, which is evidently quite constant in our series of analyses.

The present values for cytochrome *c* agree well with those previously reported from our laboratory (3, 6) for rat liver, heart, and kidney cortex. The results for liver not only confirm the earlier high values, but indeed are somewhat higher as well as more consistent. In these fasted animals, in contrast to those previously used (3, 6), solutions of isolated cytochrome *c* relatively free from turbidity were regularly obtained. This finding appears to support the conclusion of Rosenthal and Drabkin (3) that lower values for the pigment and presence of turbidity are in part attributable to the content of glycogen. As before, while analyses for the cytochrome *c* of rat heart and kidney cortex are of the same magnitude as those found by other investigators (1, 2), our results for liver are markedly higher. One of us (D. L. D.) has examined a number of rat liver extracts, prepared according to Rosenthal and Drabkin (3), in which simultaneous analyses for cytochrome *c* were carried out by a slightly modified (for adjustment to larger quantities) Potter-DuBois kidney succinic dehydrogenase technique (2) and by our direct micro spectrophotometric method, $Na_2S_2O_4$ being employed for reduction (3). Similar results were yielded by the independent analytical procedures. Hence our values for cytochrome *c* in liver, significantly higher than those reported by the Wisconsin workers (2), cannot be ascribed to differences in the spectrophotometric steps of the analytical methods.

In Tables II and III, which are self-explanatory, the data required for the estimation of the total cytochrome *c*, myoglobin, and hemoglobin of the rat (reference body weight 250 gm.) are recorded. The quantities in descending order of magnitude are hemoglobin 3190 mg., myoglobin 101 mg., and cytochrome *c* 14.4 mg. The ratio of hemoglobin to myoglobin to cytochrome *c* is hence 222:7:1 in this species. It is of interest that, while

the concentration of cytochrome *c* is high in rat tissues (Rosenthal and Drabkin (6)), the concentration of myoglobin appears to be quite low in comparison with other species (dog, Whipple (31)). Whether any special

TABLE II

*Total Cytochrome c Content of Young, Adult, Male Albino Rat, Fasted 18 to 24 Hours*

The values are calculated from analytical data for a reference body weight of 250 gm.

| Organ | Weight of organ* | Per cent of body weight | Cytochrome *c* | |
|---|---|---|---|---|
| | | | Per gm. tissue, wet weight | Total in organ |
| | *gm.* | | *γ* | *γ* |
| Skeletal muscles | 113.0 | 45.2 | 98† | 11,080 |
| Liver | 6.50 | 2.6 | 223† | 1,450 |
| Kidneys | 1.63 | 0.65 | 352† | 573 |
| Abdominal organs‡ | 20.8 | 8.3 | 18§ | 374 |
| Skin | 46.5 | 18.6 | 8§ | 372 |
| Heart | 0.73 | 0.29 | 447† | 326 |
| Brain | 1.93 | 0.77 | 82‖ | 158 |
| Blood | 20.2¶ | 8.1 | 1.3§ | 26 |
| Testes | 2.50 | 1.0 | 10.2§ | 26 |
| Lungs | 1.42 | 0.57 | 5.2‖ | 7 |
| Skeleton | 26.3 | 10.5 | ** | |
| Rest | 8.49†† | 3.4 | ** | |
| Total | 250 | 100 | | 14,392 |

* Except for the blood, based upon measurements of three male fasted rats of approximately 250 gm. each.

† Mean values of analyses reported in Table I.

‡ Exclusive of liver and kidneys.

§ Data on aliquots from pooled tissue specimens of the rats used for the determination of organ weights.

‖ Data of Rosenthal and Drabkin (6).

¶ Calculated from a plasma volume determined with the dye T1824 (28) in one animal = 9.98 ml., hematocrit = 48 per cent, and specific gravity = 1.05.

** Cytochrome *c* concentration not determined; assumed to be negligible in these tissues.

†† The difference between the total body mass of 250 gm. and the summation of the other components.

significance may be attached to this apparent reciprocal relationship of cytochrome *c* and myoglobin in the rat must await data on these relationships now being gathered for other species (dog, horse, and man) in our laboratory. Although the cytochrome *c* concentration (Table I) is highest in rat heart and kidney, the liver and muscles (Table II) contain nearly 90

per cent of the total cytochrome *c* (12.53 out of 14.39 mg.), and the muscles alone, with a relatively low concentration of the biocatalyst, contain, because of their large share of the total mass of the organism, nearly 80 per cent.

Data upon the concentration of cytochrome *c* in excised liver and in the restored liver of nine partially hepatectomized rats are presented in Table IV. Supplementary data are also given for heart, kidney cortex, and muscle of these animals. It is evident that the concentrations of cytochrome *c* in the various tissues, including liver, of rats subjected to the ac-

TABLE III

*Total Myoglobin and Hemoglobin Contents of Young, Adult, Male Albino Rat of 250 Gm. Body Mass*

| Organ | Weight of organ* | Volume of organ | Myoglobin | | Hemoglobin | |
|---|---|---|---|---|---|---|
| | | | Per gm. tissue,† wet weight | Total in organ | Per 100 ml. tissue | Total in organ |
| | *gm.* | *ml.* | *mg.* | *mg.* | *gm.* | *gm.* |
| Skeletal muscles | 113.0 | | 0.89 | 100.6 | | |
| Heart | 0.73 | | 0.91 | 0.66 | | |
| Blood | 20.2 | 19.2‡ | | | 16.6 | 3.19§ |
| Total in body | | | | 101 | | 3.19 |

* See Table II.

† Analyses by Procedure 1 (see "Experimental methods") on 80 and 6.02 gm. of pooled skeletal and cardiac muscle respectively from six rats. Individual analyses on muscle of hind limb from Rats 8, 9, and 15 (Table I) by the micro spectrophotometric technique (Procedure 2, "Experimental methods") were respectively 0.50, 0.68, and 0.57 (mean = 0.58) mg. per gm. of wet weight of tissue.

‡ Calculated from plasma volume 9.98 ml. and hematocrit 48 per cent (Table II).

§ For calculation see "Experimental methods."

tive hyperplastic process in liver, following its partial excision, were similar in magnitude to corresponding values in the controls (Table IV, n. values for liver, and Table I for the other tissues). The sex had no influence on the results. The restored liver had a slightly, but significantly, lower water content ($W:D$ ratios, Column 5) than that of corresponding excised (control) liver tissue. While the difference in the means of the $W:D$ values for excised and restored liver is only $-0.30$ ($3.55 - 3.25$), this difference is statistically significant. Tested by Fisher's method (32), the individual differences in $W:D$ ratios yield a value of $t = 0.30/0.072$ (the standard error of mean differences) $= 4.16$. This value corresponds in Fisher's table of $t$ (32) to a probability, $P$, of less than 0.01 that the difference in the means

is a matter of chance. Deviating in the opposite direction from the water content was the protein-bound phosphorus (Column 6, Table IV), which

TABLE IV

*Concentration (C) of Cytochrome c in Liver and Other Tissues of Rats Subjected to Partial Hepatectomy*

| Rat No.* (1) | Sex (2) | Body weight (3) | Liver | | | Heart | | Kidney cortex | | Skeletal muscle | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C, wet weight (4) | W : D† (5) | P,‡wet weight (6) | C, wet weight (7) | W : D (8) | C, wet weight (9) | W : D (10) | C, wet weight (11) | W : D (12) |
| | | *gm.* | *γ per gm.* | | *γ per gm.* | *γ per gm.* | | *γ per gm.* | | *γ per gm.* | |
| H1 (n.) | F. | 184 | 96 | 3.77 | 921 | | | | | | |
| H1 (r.) | | 196 | 187 | 3.48 | 950 | 713 | 4.74 | 294 | 3.79 | 124 | 3.98 |
| H2 (n.) | " | 190 | 159 | 3.46 | 839 | | | | | | |
| H2 (r.) | | 187 | 214 | 3.15 | 1020 | 468 | 4.48 | 293 | 4.05 | 83 | 3.99 |
| H3 (n.) | " | 198 | 131 | 3.38 | 1005 | | | | | | |
| H3 (r.) | | 191 | 154 | 3.01 | 1130 | 360 | 3.98 | 228 | 4.40 | 101 | 4.00 |
| H4 (n.) | " | 201 | 105 | 3.34 | 704 | | | | | | |
| H4 (r.) | | 201 | 191 | 3.14 | 1130 | 496 | | 309 | 3.65 | 119 | 4.03 |
| H5 (n.) | M. | 267 | (218) | 3.68 | 1012 | | | | | | |
| H5 (r.)§ | | 273 | (174) | | 1002 | 381 | 4.20 | 371 | 4.01 | 125 | 3.99 |
| H6 (n.) | " | 268 | 180 | | 891 | | | | | | |
| H6 (r.)§ | | 281 | 184 | 3.36 | 1090 | | 4.21 | 403 | 4.37 | 113 | 4.18 |
| H7 (n.) | " | 221 | 181 | 4.01 | 1020 | | | | | | |
| H7 (r.)§ | | 247 | 203 | 3.33 | 979 | 392 | 4.96 | 375 | 4.23 | 90 | 4.07 |
| H8 (n.) | " | 288 | 268 | 3.43 | 1060 | | | | | | |
| H8 (r.) | | 275 | 265 | 3.22 | 1120 | 385 | 4.58 | 457 | 4.24 | 117 | 3.99 |
| H9 (n.) | " | 332 | 290 | 3.37 | 981 | | | | | | |
| H9 (r.) | | 321 | 289 | 3.30 | 1090 | 394 | 4.22 | 386 | 3.94 | 121 | 4.05 |
| Mean ± s.e.‖ (n.) | | 239 | 176 ±25 | 3.55 ±0.06 | 937 ±37 | | | | | | |
| Mean ± s.e. (r.) | | 241 | 211 ±16 | 3.25 ±0.05 | 1057 ±23 | 449 ±41 | 4.42 ±0.12 | 346 ±23 | 4.08 ±0.09 | 110 ±5 | 4.03 ±0.02 |

* The letters in parenthesis, (n.) and (r.), designate respectively the analyses on the excised liver ("internal controls"), and on the tissues of the same rats 14 days postoperatively (full restoration of liver).

† $W : D$ = wet weight to dry weight ratios.

‡ P = protein-bound phosphorus (6).

§ Rats H5, H6, and H7 were on the enriched protein diet; the other rats were on the stock diet (see foot-note 4).

‖ Standard error = $\sqrt{\Sigma d^2/n(n-1)}$. The values (in parentheses) for cytochrome *c* in the liver of Rat H5 were excluded in calculating the means, since the analysis for cytochrome *c* in the restored liver of this animal (r. value) is regarded to be faulty (see the text).

was in seven out of nine rats somewhat higher in the restored liver. The difference in the mean values for protein-bound phosphorus of restored and

excised liver was +120 (1057 − 937). Tested by Fisher's method, this difference was found to be within the border line of statistical significance, with $t = 120/47 = 2.56$, and $P$ = less than 0.05. Hence it must be concluded from the protein-bound phosphorus analyses that the restored liver, even after 14 days of regeneration, had a slightly higher cellularity (6) than the original liver tissue. It may be noted that the cytochrome *c* concentration (Column 4) shows a change in a direction similar to that of the protein-bound phosphorus. In only one animal, Rat H5, is the value for cytochrome *c* concentration in the restored liver appreciably lower than in the excised tissue. This result is probably analytically erroneous. When the results on this rat are excluded, the difference in means for cytochrome *c* in restored and excised liver is +35 (211 − 176) and is significant, according to Fisher's criterion ($t = 2.59$, $P$ = less than 0.05). It also appears significant that the mean values for cytochrome *c* concentration per microgram of protein-bound phosphorus are similar in restored and excised liver (211/1057 = 0.200, and 176/937 = 0.188 $\gamma$ respectively).

The following values with their standard errors illustrate the constancy of structural relationships of the liver in the young adult rats of our colony. In four fed females, weight of individuals from 254 to 283 gm., the total liver = 3.13 ± 0.10 per cent of the body weight, while the left lateral lobe = 34.4 ± 0.7, the median lobe = 33.7 ± 0.8, the right lateral and caudal lobes = 31.9 ± 0.9, and the left lateral plus the median lobe = 68.1 ± 0.9 per cent of the liver mass. In animals fasted 18 to 24 hours (ten males and two females), weight of individuals from 182 to 357 gm., the total liver = 2.62 ± 0.04 per cent of the body weight, while the left lateral lobe = 36.2 ± 0.2, the median lobe = 32.2 ± 0.2, the right lateral and caudal lobes = 31.6 ± 0.2, and the left lateral plus the median lobe = 68.4 ± 0.2 per cent of the liver weight. It may be noted that fasting for 18 to 24 hours markedly altered the percentage liver of body mass, and the standard errors suggest that appreciably closer agreement of values was obtained in the fasted animals. This is not surprising, since variations are recognized to exist in storage substances of growing animals fed *ad libitum*.[5] In fasted rats our value for the percentage mass of the left lateral plus the median lobe is in excellent agreement with that reported by Brues *et al.* (12), who concluded that the relationship of total liver to individual lobes is a safer base-line to employ in the interpretation of the quantity of regeneration than is the ratio of body mass to liver mass.

In Table V calculations, based on the data in Table IV and those given above in the text, are recorded for the quantity of new cytochrome *c* which has appeared in the regenerating liver in a postoperative period of 14 days.

[5] Dr. H. M. Vars has found (personal communication to us) that in similar rats fasted for 18 to 24 hours the glycogen is low (of the order of 0.5 to 0.6 per cent) and quite constant.

It is obvious from the values in Table V that from our data the same conclusions could be derived whether the weight of the original liver was taken

TABLE V

*Quantity of New Cytochrome c in Rat Liver after 14 Days of Restoration*

| Rat No. | Body weight (initial) | Amount of liver excised | Weight of original liver (4) | | Amount of remaining liver (5) | | Cytochrome *c* in remaining liver‡ (6) | | Weight of restored liver | Total pigment§ | Cytochrome *c* in restored liver New pigment‖ (9) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | (*a*) | | (*b*) | |
| (1) | (2) | (3) | (*a*)* | (*b*)† | (*a*) | (*b*) | (*a*) | (*b*) | (7) | (8) | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *γ* | *γ* | *gm.* | *γ* | *γ* | *per cent* | *γ* | *per cent* |
| H1 | 184 | 2.47 | 4.82 | 3.61 | 2.35 | 1.14 | 226 | 109 | 6.01 | 1123 | 897 | 79.8 | 1014 | 90.2 |
| H2 | 190 | 3.01 | 4.98 | 4.40 | 1.97 | 1.39 | 313 | 221 | 4.01 | 858 | 545 | 63.5 | 637 | 74.2 |
| H3 | 198 | 1.76 | 5.19 | 4.86 | 3.43 | 3.10 | 449 | 406 | 3.61 | 556 | 107 | 19.2 | 150 | 27.0 |
| H4 | 201 | 1.52 | 5.27 | 4.20 | 3.78 | 2.68 | 397 | 282 | 5.38 | 1028 | 631 | 61.4 | 746 | 72.6 |
| H5 | 267 | 2.65 | 7.00 | 7.32 | 4.35 | 4.67 | 949 | 1020 | 6.14 | 1069 | 120 | 11.2 | 49 | 4.6 |
| H6 | 268 | 2.94 | 7.03 | 8.12 | 4.09 | 5.18 | 737 | 933 | 6.93 | 1275 | 538 | 42.2 | 342 | 26.8 |
| H7 | 221 | 2.42 | 5.79 | 6.68 | 3.37 | 4.26 | 610 | 771 | 5.65 | 1147 | 537 | 46.8 | 376 | 32.8 |
| H8 | 288 | 2.84 | 7.55 | 7.57 | 4.71 | 4.73 | 1262 | 1268 | 5.99 | 1588 | 326 | 20.5 | 320 | 20.2 |
| H9 | 332 | 3.11 | 8.70 | 8.59 | 5.59 | 5.48 | 1621 | 1590 | 7.51 | 2170 | 549 | 25.3 | 560 | 25.8 |
| Mean¶ | | | | | | | | | | 1313 | 575 | 48.5 | 571 | 48.9 |
| Rate, per day, of appearance of new cytochrome *c* | | | | | | | | | | | 41 | 3.5 | 41 | 3.5 |

* Values in columns labeled (*a*) calculated on the basis of the weight of the original liver = 2.62 per cent of body weight (see the text and Column 2, this table).

† Values in columns labeled (*b*) calculated on the basis of percentage of liver tissue excised (from the relationship of lobes to total liver given in the text). In Rats H1 and H2 both the left lateral and the median lobes were removed, or 68.4 per cent was excised (see the text); in the remaining animals only the left lateral lobe (= 36.2 per cent) was removed.

‡ Calculated by multiplying the values for concentration of cytochrome *c* in excised liver (n. values in Table IV, Column 4) by the corresponding weights of remaining liver tissue (Column 5, this table).

§ Calculated by multiplying the values for the concentration of cytochrome *c* in restored liver (r. values in Table IV, Column 4) by the corresponding weights of the total restored liver (Column 7, this table).

‖ Total pigment (Column 8) minus corresponding values for cytochrome *c* in remaining liver tissue (Column 6).

¶ Data from Rats H3 and H5 were excluded in calculating the means. In Rat H3 the amount of restoration of liver was abnormally low; in Rat H5 the analytical value for cytochrome *c* concentration in the restored liver is thought to be erroneous (see the text).

as a fixed percentage of body weight (columns (*a*)) or was based on a definite relationship of individual lobes to total liver (columns (*b*)). The rate of appearance of new cytochrome *c* in the regenerating liver was found to

average 41 $\gamma$ per day. This was equivalent to a daily increment in pigment of 3.5 per cent, and was of the same magnitude (percentage basis) as the increase in new liver tissue. For unknown reasons, in one rat (No. H3) appreciable restoration of the liver failed to occur. In another animal (Rat H1) the restored liver had an appreciably larger weight than that calculated for the original liver. This phenomenon of the regeneration process has been observed previously (11).

## DISCUSSION

The technique of partial hepatectomy may serve not only for the study of the metabolism of tissue constituents (as in the present work), but also in the elucidation of cellular regeneration, a phenomenon of the utmost importance. For both purposes an obvious approach is a study of the influence of the diet. In the experiments reported here (Tables IV and V) dietary considerations were minor. No difference was observed on an adequate protein (20 per cent) and on an enriched protein (32 per cent) diet, either in the rate of regeneration or in the rate of appearance of new cytochrome *c*.

In describing the phenomenon of increase in total cytochrome *c* of liver which occurred in the process of tissue restoration, we have used the phrase *rate of appearance of new cytochrome c in liver* rather than *rate of production of cytochrome c*. We did not employ the latter description since we wished to avoid the suggestion that the present experiments have demonstrated a production *de novo* of cytochrome *c* in the body. It may be that cytochrome *c* had been manufactured from its building stones in the newly formed liver tissue. There are indirect arguments in favor of this possibility. (1) The concentration of cytochrome *c* and the cellularity (protein-bound phosphorus, discussed above) are proportional. (2) The concentration of the pigment is similar in restored and in normal, adult liver. This similarity of concentration in regenerated and normal liver has been found (17) to hold also for arginase, an exclusive constituent of liver. (3) No evidence exists that a protein such as cytochrome *c* can migrate from one tissue to another. On the other hand, cytochrome *c* is an unusual protein of low molecular weight (21), with a universal distribution in the cells of all aerobic tissues. While an appreciable amount (571 $\gamma$, Table V) of new cytochrome *c* had appeared in the liver in 14 days of restoration, and while the concentration of cytochrome *c* in heart, kidney, and skeletal muscle had remained unaltered following partial hepatectomy (compare the values in Tables I and IV), it may be questioned whether the new pigment in liver had not been preformed in another tissue. The only tissue from which cytochrome *c* could have been mobilized, without detection by our analytical procedure, is the skeletal muscle. The latter contains a total of 11,080

$\gamma$ of cytochrome *c* (Table II). The amount of new pigment in liver is only 5.2 per cent ((571 $\times$ 100)/11,080) of the above quantity. Special techniques will be required to settle the issue whether cytochrome *c* can be transferred from one tissue to another.

The rate of appearance (41 $\gamma$ per day, or a daily increment of 3.5 per cent, Table V) of cytochrome *c* in regenerating liver is not small on a percentile basis when compared to the rate of production of hemoglobin with Whipple's depletion technique (8). Hemoglobin formation under the stimulus of constant blood withdrawal is of the order of 1 gm. per kilo per day (33). Assuming a blood volume of 8 per cent and a hemoglobin concentration of 20 gm. per 100 ml. of blood (8), the percentage of new hemoglobin formed per day in Whipple's dogs is 6.3. Our percentage of 3.5 for the daily increment in cytochrome *c* in regenerating liver, therefore, is not materially different in magnitude from the percentile daily increment in hemoglobin in animals kept depleted of the blood pigment. It is also probable that higher values than 3.5 per cent might have been obtained had analyses been performed in the first few days after liver excision, when the rate of regeneration is appreciably greater (12, 13, 15).

Special attention may be called to the protein-bound phosphorus index of "cellularity." Rosenthal and Drabkin (6, 34) applied this index to the determination of the concentration of cytochrome *c*, and found that the concentration of cytochrome *c* in malignant tissues deviated most markedly from that of normal tissues when the concentration of the pigment was expressed per unit of protein-bound phosphorus (6, 34). In the case of neoplastic growths increased cellularity was accompanied by decreased cytochrome *c* concentration. This is in striking contrast with the hyperplastic process which follows partial liver excision. Here we have found small though significant increases in cytochrome *c* concentration and in protein-bound phosphorus, but both quantities have the same relationship to each other in the restored and in the excised, control liver tissue. During the course of our studies it was reported by Raska (35) that a material increase in cytochrome *c* concentration occurred in the remaining, hypertrophied kidney after unilateral nephrectomy in dogs. However, when Raska's values are related to protein-bound phosphorus, which he also determined, the change in cytochrome *c* concentration does not appear significant. Thus, the changes in cellularity which accompany the processes of hyperplasia and hypertrophy seem to differ strikingly from the neoplastic process in regard to the relationship of cytochrome *c* and the protein-bound phosphorus. The possible use of the protein-bound phosphorus as a chemical index of malignancy appears worthy of further investigation.

We are indebted to Dr. Otto Rosenthal of the Harrison Department of Surgical Research for carrying out the analyses of protein-bound phos-

phorus, and to the above department for the use of their surgical facilities. We are also grateful to Miss Ann Claire Barnett for technical help in some of the determinations of myoglobin.

## SUMMARY

Partial hepatectomy in the rat has been suggested as a useful, reproducible technique in the investigation of the metabolism of tissue constituents.

By the above means the cytochrome *c* in the regenerating liver has been studied. In a postoperative period of 14 days, allowing complete restoration of tissue, the total increase in new cytochrome *c* was 571 $\gamma$, or a daily increment of 41 $\gamma$. The rate of appearance of cytochrome *c* in the regenerating liver was 3.5 per cent per day. This rate compares favorably on a percentile basis with that of the production of hemoglobin in dogs depleted by hemorrhage (8, 33).

In the appraisal of the results in liver supplementary data were obtained on the cytochrome *c* and water contents of other tissues, heart, kidney cortex, and skeletal muscle.

The protein-bound phosphorus as an index of cellularity (6) was determined in the excised and restored liver tissue, and its value in the interpretation of the findings has been discussed.

Sufficient analytical data have been accumulated to permit a reliable estimation of the total cytochrome *c*, myoglobin, and hemoglobin of a young, adult albino rat (reference body weight 250 gm.). The quantities in descending order were hemoglobin 3190 mg., myoglobin 101 mg., and cytochrome *c* 14.4 mg. The ratio of these main hemin derivatives is therefore 222:7:1 in this species.

## BIBLIOGRAPHY

1. Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
2. Potter, V. R., and DuBois, K. P., *J. Biol. Chem.*, **142**, 417 (1942).
3. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
4. Fujita, A., Hata, T., Numata, I., and Ajisaka, M., *Biochem. Z.*, **301**, 376 (1939).
5. Junowicz-Kocholaty, R., and Hogness, T. R., *J. Biol. Chem.*, **129**, 569 (1939).
6. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **150**, 131 (1943).
7. Greenstein, J. P., Werne, J., Eschenbrenner, A. B., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **5**, 55 (1944).
8. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **115**, 651 (1936).
9. Fishback, F. C., *Arch. Path.*, **7**, 955 (1929).
10. Ponfick, E., *Verhandl. deut. Ges. Chir.*, **19**, 28 (1890).
11. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, **12**, 186 (1931).
12. Brues, A. M., Drury, D. R., and Brues, M. C., *Arch. Path.*, **22**, 658 (1936).
13. Brues, A. M., and Marble, B. B., *J. Exp. Med.*, **65**, 15 (1937).
14. Marshak, A., and Walker, A. C., *Am. J. Physiol.*, **143**, 226, 235 (1945).
15. Norris, J. L., Blanchard, J., and Povolny, C., *Arch. Path.*, **34**, 208 (1942).
16. Robertson, W. von B., and Kahler, H., *J. Nat. Cancer Inst.*, **2**, 595 (1942).

17. Greenstein, J. P., Edwards, J. E., Andervant, H. B., and White, J., *J. Nat. Cancer Inst.*, **3**, 7 (1942). Greenstein, J. P., American Association for the Advancement of Science, Research conference on cancer, Washington, 192 (1945).
18. Greenstein, J. P., and Thompson, J. W., *J. Nat. Cancer Inst.*, **4**, 271 (1944).
19. Young, G., and Wald, G., *Am. J. Physiol.*, **131**, 210 (1940–41).
20. Chanutin, A., Hortenstine, J. C., Cole, W. S., and Ludewig, S., *J. Biol. Chem.*, **123**, 247 (1938).
21. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1804 (1941).
22. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
23. Morgan, V. E., *J. Biol. Chem.*, **112**, 557 (1935–36). Theorell, H., *Biochem. Z.*, **252**, 1 (1932); **268**, 46 (1934).
24. Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).
25. Drabkin, D. L., *J. Biol. Chem.*, **146**, 605 (1942).
26. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 51 (1935–36).
27. Drabkin, D. L., and Fitz-Hugh, T., Jr., *Am. J. Physiol.*, **108**, 61 (1934).
28. Gregersen, M. I., Gibson, J. J., and Stead, E. A., *Proc. Am. Physiol. Soc.*, *Am. J. Physiol.*, **113**, 54 (1935). Gibson, J. G., 2nd, and Evans, W. A., Jr., *J. Clin. Invest.*, **16**, 301 (1937).
29. Steenbock, H., *Science*, **58**, 449 (1923).
30. McBride, J. J., Guest, M. M., and Scott, E. L., *J. Biol. Chem.*, **139**, 943 (1941).
31. Whipple, G. H., *Am. J. Physiol.*, **76**, 693 (1926).
32. Fisher, R. A., Statistical methods for research workers, London, 4th edition, 151 (1932).
33. Robscheit-Robbins, F. S., Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, **82**, 311 (1945).
34. Rosenthal, O., and Drabkin, D. L., *Cancer Res.*, **4**, 487 (1944).
35. Raska, S. B., *J. Exp. Med.*, **82**, 227 (1945).